

Fanconi Anemia Complementation Group A Cells Are Hypersensitive to Chromium(VI)-Induced Toxicity

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Fanconi anemia (FA) is an autosomal recessive disorder characterized by diverse developmental abnormalities, progressive bone marrow failure, and a markedly increased incidence of malignancy. FA cells are hypersensitive to DNA cross-linking agents, suggesting a general defect in the repair of DNA cross-links. Some forms of hexavalent chromium [Cr(VI)] are implicated as respiratory carcinogens and induce several types of DNA lesions, including ternary DNA–Cr–DNA interstrand cross-links (Cr–DDC). We hypothesized that human FA complementation group A (FA-A) cells would be hypersensitive to Cr(VI) and Cr(VI)-induced apoptosis. Using phosphatidylserine translocation and caspase-3 activation, human FA-A fibroblasts were found to be markedly hypersensitive to chromium-induced apoptosis compared with CRL-1634 cells, which are normal human foreskin fibroblasts (CRL). The clonogenicity of FA-A cells was also significantly decreased compared with CRL cells after Cr(VI) treatment. There was no significant difference in either Cr(VI) uptake or Cr–DNA adduct formation between FA-A and CRL cells. These results show that FA-A cells are hypersensitive to Cr(VI) and Cr-induced apoptosis and that this hypersensitivity is not due to increased Cr(VI) uptake or increased Cr–DNA adduct formation. The results also suggest that Cr–DDC may be proapoptotic lesions. These results are the first to show that FA cells are hypersensitive to an environmentally relevant DNA cross-linking agent. **Key words:** apoptosis, carcinogen, caspase-3, clonogenicity, DNA adducts, genotoxin, phosphatidylserine translocation, sodium chromate, uptake. *Environ Health Perspect* 110(suppl 5):773–777 (2002).

<http://ehpnet1.niehs.nih.gov/docs/2002/suppl-5/773-777/vilcheck/abstract.html>

Certain chromium compounds are well-established human respiratory toxins and carcinogens for which adverse health effects are usually associated with occupational exposure (1). Epidemiologic studies carried out in Europe, Japan, and the United States have consistently shown that workers in the chromate production industry have an elevated risk of respiratory disease, perforation of the nasal septum, development of nasal polyps, and lung cancer (2,3). Certain inhaled particulate chromium compounds may persist in the lungs and cause long-term effects. Bifurcations of the lung bronchi of chromate workers have been found to contain millimolar concentrations of chromium more than two decades after cessation of exposure (4). The deposition of chromium in industrial waste, either in the form of dissolved chromium released to surface waters or chromate slag used in landfills, has also raised concerns about chromium as a potential environmental hazard (5). Investigators have identified more than 160 chromate production waste sites within Hudson County, New Jersey, distributed throughout the community in both industrial and residential areas. Frequently, the waste material contained levels of hexavalent chromium in the tens of parts per million (ppm) and sometimes in excess of 100 ppm (6).

Epidemiologic, animal, and *in vitro* cell studies have consistently shown that the

hexavalent form of certain particulate chromium compounds [Cr(VI)] is the most important toxic and carcinogenic species (1). The main targets of Cr(VI) toxicity are lung epithelial cells and fibroblasts exposed to high concentrations of soluble Cr(VI) in the immediate microenvironment of inhaled particles (7). Soluble Cr(VI) compounds are genotoxic (8–11) and can induce gene mutations (12), sister chromatid exchanges (8,13), and chromosomal aberrations (14,15). In addition, Cr(VI) produces a variety of DNA lesions including DNA single-strand breaks, DNA–protein cross-links (DPC), Cr–DNA adducts, and ternary DNA–Cr–DNA cross-links (Cr–DDC) (16–25). At least one of these forms of structural DNA damage, the Cr–DDC, results in functional damage in the form of polymerase-arresting lesions (26,27). Thus, soluble sodium chromate (Na₂CrO₄) can be used to study the genotoxic and cytotoxic effects of Cr(VI) in cell culture.

If a cell sustains a genotoxic insult, the damage must be repaired or bypassed before replication to prevent death or the outgrowth of cells with potentially oncogenic alterations. If the damage is irreparable, the cell must be removed from the proliferating population. The predominant cellular fates in response to irreparable DNA damage are terminal growth arrest and apoptosis (28,29). Cell populations exposed to Cr(VI) have different spectrums of

responses, depending on the extent of DNA damage (29).

Fanconi anemia (FA) is an autosomal recessive disorder characterized by diverse developmental abnormalities, progressive bone marrow failure, and a markedly increased incidence of malignancy. FA cells are hypersensitive to the DNA cross-linking agents diepoxybutane, cisplatin, and mitomycin C, suggesting a general defect in the repair of DNA cross-links (30–34). Eight genetic complementation groups have been described (FA-A through FA-H), with group A accounting for 60–65% of FA patients (30). FA-A cells are deficient in the repair of DNA interstrand cross-links (35); thus, FA-A fibroblasts were employed in this study. Our intent was to test the hypothesis that human FA-A cells would display an increased sensitivity to Cr(VI)-induced cell death. The data suggest that the FA-A gene (*FANCA*) is essential for survival after exposure to Cr(VI).

Materials and Methods

Cell Culture

CRL cells (American Type Culture Collection CRL-1634 cells) are normal human foreskin fibroblasts isolated from a newborn black male. FA-A cells (Coriell Cell Repositories GM01309) are FA-A human fibroblasts isolated from a black male 12 years of age. Both CRL and FA-A cells were maintained in minimal essential medium Eagle-Earle media (Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT, USA), 2× essential and nonessential amino acids, vitamins, and 2 mM L-glutamine

This article is part of the monograph *Molecular Mechanisms of Metal Toxicity and Carcinogenicity*.

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This work was conducted in partial fulfillment of the requirements for the PhD degree in molecular and cell oncology, Columbian Graduate School of Arts and Sciences, The George Washington University, Washington, DC.

This work was supported by grants NIH ES 05304 and ES 09961 (to S.R.P.) from the National Institutes of Health.

Received 11 February 2002; accepted 20 May 2002.

(Gibco). Cells were incubated in a 95% air, 5% CO₂ humidified atmosphere at 37°C.

Chromium Preparation

Sodium chromate (Na₂CrO₄ × 4H₂O) (J.T. Baker Chemical Co., Phillipsburg, NJ, USA) was dissolved in double-distilled water and sterilized through a 0.2-μm filter before use.

Phosphatidylserine Translocation

The phosphatidylserine (PS) translocation assay was used to investigate the sensitivity of FA-A and CRL cells to Cr-induced apoptosis. This assay measures PS translocation from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (extracellular) leaflet in the early stages of apoptosis. Annexin V protein has a strong, specific affinity for PS (36), and PS on the outer leaflet is available for binding labeled Annexin V. Positive cells exhibit green fluorescence around the plasma membrane. CRL and FA-A cells were seeded at 10⁵ cells/60-mm dish and incubated for 24 hr prior to Cr(VI) exposure. Cells were treated with a final concentration of 0, 1, 3, 6, or 7 μM Cr(VI) for 24 hr in complete media. After 24 hr, the cells were rinsed twice with 1× phosphate-buffered saline (PBS) and incubated for an additional 24 hr in fresh media before analysis. Cells were gently harvested by trypsinization, combined with non-adherent cells from the culture medium, and centrifuged at 600×g for 5 min. Cell pellets were washed once in 1× PBS and resuspended in 100 μL binding buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂] containing 2 μL Annexin(V)-FLUOS (Roche, Indianapolis, IN, USA). Samples were incubated in the dark at room temperature for 15 min. Thirty microliters of each sample was loaded on a microscope slide and the percentage of Annexin(V)-FLUOS-stained cells was determined by counting five fields that contained at least 30 cells each on an Olympus AX70 microscope (Olympus, Lake Success, NY, USA) with a fluorescent filter set suitable for FLUOS analysis (excitation at 460–490 nm and emission at 515 nm).

Caspase-3 Activity

Caspase-3 is derived from the proenzyme CPP32 at the onset of apoptosis and plays a pivotal role in programmed cell death (37–40). Caspase-3 exhibits the highest similarity to *C. elegans* cell death gene of the ICE (interleukin-1β-converting enzyme) proteases (41). Therefore, caspase-3 is an excellent biochemical indicator of apoptosis. The caspase-3 fluorescent assay detects a shift in fluorescence emission of 7-amino-4-trifluoromethyl coumarin (AFC). AFC is conjugated to a specific tetrapeptide sequence that normally emits blue fluorescence. After the substrate is

cleaved by caspase-3, the liberated AFC emits a yellow-green fluorescence at 505 nm. Nearly confluent 150-cm² flasks were passaged at a 1:3 ratio and incubated for 24 hr prior to Cr(VI) exposure. CRL and FA-A cells were treated with a final concentration of 0, 1, 3, 6, or 7 μM Cr(VI) for 24 hr in complete media. After 24 hr the cells were rinsed twice with 1× PBS, the media was replaced, and the cells were incubated for an additional 24 hr before analysis. Cells were harvested by cell scraping and combined with nonadherent cells from the culture medium. Caspase-3 activity was determined using the FluorAce Apopain Assay Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's protocol. Cells were centrifuged at 600×g for 5 min. Cell pellets were rinsed once in 1× PBS and resuspended in 100 μL ice-cold apopain lysis buffer [10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), supplemented with protease inhibitors (10 μg/mL pepstatin A, 10 μg/mL aprotinin, 20 μg/mL leupeptin) (Sigma, St. Louis, MO, USA)]. Cell suspensions were vortexed gently, then freeze/thawed 4 times by transferring from an isopropanol-dry ice bath to a 37°C water bath. Cell suspensions were then centrifuged at 12,000×g for 30 min at 4°C to pellet cell debris. Protein concentrations of the resulting supernatants were determined with the DC Protein Assay II (Bio-Rad). Samples containing an equal amount of protein were diluted to 1 mL and transferred to wells of a CoStar 48-well dish (Corning, Corning, NY, USA). Ten microliters of apopain substrate (Ac-DEVD-AFC) (Bio-Rad kit) and 40-μL 25× reaction buffer (Bio-Rad kit) were added to each sample and control. Caspase-3 enzymatically cleaves the AFC from the peptide and releases free AFC, which then produces a blue-green fluorescence. The samples were gently mixed, and the fluorescence was determined using a Cytofluor 4000 fluorescence multiwell plate reader (PE Biosystems, Foster City, CA, USA) (excitation at 320–400 nm and emission at 505–555 nm). Fluorescence readings were taken at $t = 0$, $t = 30$, $t = 60$, $t = 90$, and $t = 120$ min after addition of substrate. The amount of AFC released from the substrate was determined by linear regression of an AFC standard curve (per manufacturer's instructions) at the time point at which the apopain positive control showed the greatest increase. Caspase-3 activity was determined and expressed as the ΔAFC/min.

Clonogenicity

CRL and FA-A cells were seeded at 10⁵ cells/100-mm dish and incubated for 24 hr prior to Cr(VI) exposure. Cells were treated

with a final concentration of 0, 0.01, 0.1, 0.5, 1, 2, or 3 μM Cr(VI) for 24 hr in complete media. Cells were washed twice with 1× PBS, collected by trypsinization, counted, and reseeded at 2,000 cells/100-mm dish in triplicate. The plates were incubated for 7–8 days and then rinsed with 1× PBS and incubated with crystal violet stain (80% methanol, 2% formaldehyde, and 2.5 g/L crystal violet) for 15 min at room temperature. The plates were thoroughly rinsed with distilled water and allowed to dry. Colonies were counted and the means ± SE of triplicate cultures were used to determine clonogenic survival as a percentage of control cultures.

Chromium Uptake

CRL and FA-A cells were seeded at 3 × 10⁵ cells/100-mm dish and incubated for 24 hr prior to Cr(VI) exposure. Three extra dishes of each cell type were seeded for determining final cell number. Cr(VI) was prepared as above and spiked with Na₂⁵¹CrO₄ (ICN, Irvine, CA). CRL and FA-A cells were treated with a final concentration of 0, 3, or 7 μM Cr(VI) for 3 hr at 37°C. Following Cr(VI) treatment, cells were harvested by trypsinization and centrifuged at 300×g for 5 min at 4°C. Cell pellets were washed twice in 1× PBS and lysed in 500 μL lysis buffer (10 mM Tris-Cl, 0.5% sodium dodecyl sulfate [SDS], 0.5% Triton X-100). One hundred microliters of each sample was combined with Ecolite scintillation cocktail (ICN, Irvine, CA, USA). Disintegrations per minute (DPM) were determined on a Beckman LS3801 scintillation counter (Beckman Instruments, Fullerton, CA, USA). Final cell number was determined in replicate dishes on a Coulter cell counter (Coulter, Louton, UK). Data were normalized to cell number.

Chromium–DNA Adducts

Cr(VI) was prepared as above and spiked with Na₂⁵¹CrO₄ (ICN). CRL and FA-A cells were treated with 0, 3, or 7 μM Cr(VI) for 2 hr at 37°C. Following Cr(VI) treatment, cells were harvested by trypsinization and centrifuged at 300×g for 5 min at 4°C. Cell pellets were washed twice in 1× PBS and lysed in 500 μL lysis buffer (10 mM Tris-Cl, 0.5% SDS, 0.5% Triton X-100) containing 20 mg/mL proteinase K overnight in a 55°C water bath. Samples were then incubated with 10 mg/mL RNase for 4 hr in a 37°C water bath. DNA was extracted from the cell lysates, using phenol-chloroform extraction and ethanol precipitation. The DNA was then quantified by its spectrophotometric absorbance at 260 nm. DNA-bound ⁵¹Cr was quantified by scintillation counting of an aliquot of each sample in EcoLite scintillation cocktail (ICN). DPM were then used to calculate Cr–DNA adducts per 10,000 DNA base pairs.

Statistics

Statistical significance was calculated using the Student *t*-test. Significance was defined as $p < 0.05$.

Results

Cr(VI) caused a dose-dependent increase in the number of Annexin V–positive FA-A cells (Figure 1). The percent apoptosis in the CRL cells, as determined by PS translocation, was 12.1 and 16.4% after treatment with 6 and 7 μM Cr(VI), respectively, for 24 hr followed by a 24-hr recovery period. This is similar to results shown in a previous report using human lung fibroblasts (42). In comparison, the percentage of apoptotic FA-A cells was significantly more than that of CRL cells at 43.9 and 52.4% at 6 and 7 μM doses, respectively.

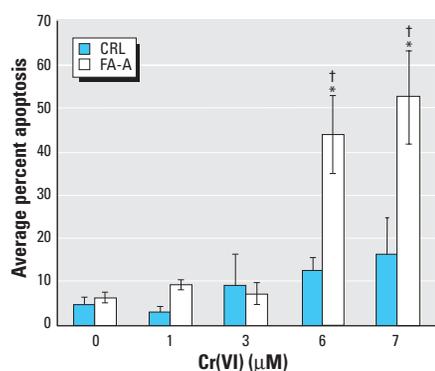


Figure 1. Comparison of Cr(VI)-induced apoptosis in CRL and FA-A cells. CRL and FA-A cells were exposed to 0, 1, 3, 6, or 7 μM Na_2CrO_4 for 24 hr. Samples were analyzed for percentage of apoptotic cells by PS translocation. Data represent an average (\pm SE) of three independent experiments, each done in triplicate. Asterisk (*) indicates a statistically significant difference ($p < 0.05$) relative to vehicle control. Cross (†) indicates a statistically significant difference ($p < 0.05$) between FA-A and CRL cells.

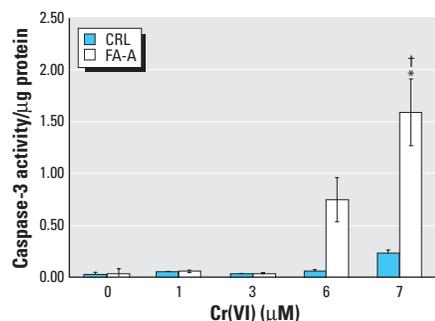


Figure 2. Comparison of Cr(VI)-induced caspase-3 activation in CRL and FA-A cells. CRL and FA-A cells were exposed to 0, 1, 3, 6, or 7 μM Na_2CrO_4 for 24 hr. Samples were analyzed for caspase-3 activity using the Fluorace Apopain Assay Kit (Bio-Rad). Data represent an average (\pm SE) of two independent experiments, each done in triplicate. Asterisk (*) indicates a statistically significant difference ($p < 0.05$) relative to vehicle control. Cross (†) indicates a statistically significant difference ($p < 0.05$) between FA-A and CRL cells.

To confirm the hypersensitivity of FA-A cells to Cr(VI)-induced apoptosis using a biochemical assay, we measured caspase-3 activity of extracts from Cr(VI)-treated CRL and FA-A cells. Cr(VI) caused a dose-dependent increase in caspase-3 activation in the FA-A cells (Figure 2). The FA-A cells again showed significantly more apoptosis than the CRL cells at 6 and 7 μM Cr(VI). The caspase-3 activity/ μg protein in the CRL cells was 0.06 and 0.22 units after treatment with 6 or 7 μM Cr(VI), respectively, for 24 hr, followed by a 24-hr recovery period. In comparison, caspase-3 activity/ μg protein in the FA-A cells was 0.75 and 1.59 units at 6 and 7 μM doses, respectively (12- to 14-fold increase).

Clonogenicity is an indicator of long-term cell survival and replicative potential after

exposure to a toxic agent. Cr(VI) caused a dose-dependent decrease in clonogenic survival in both the CRL and the FA-A cells (Figure 3). The FA-A cells showed significantly less clonogenic survival after 1, 2, and 3 μM Cr(VI) treatment compared with CRL cells. The percent clonogenic survival for 1, 2, and 3 μM Cr(VI) was 28.7, 5.4, and 0.2% for the FA-A cells compared with 89.6, 18.6, and 4.4% for the CRL cells, respectively.

We performed Cr(VI) uptake analysis to determine if the differences in apoptosis and clonogenicity could be explained by differential Cr(VI) uptake. There was a dose-dependent increase in Cr(VI) uptake from 0 through 7 μM Cr(VI) (Figure 4). However, there was no significant difference in Cr(VI) uptake between CRL and FA-A cells at any of the concentrations tested. Three and 7 μM Cr(VI) resulted in approximately 8.5×10^{-4} and 1.4×10^{-3} nmol Cr per cell, respectively.

We also measured total Cr–DNA binding in both the CRL and FA-A cells to determine if the differences in apoptosis and clonogenicity could be explained by differences in Cr–DNA adduct formation. Although Cr(VI) caused a dose-dependent increase in Cr–DNA adduct formation in both CRL and FA-A cells (Figure 5), there were no significant differences in Cr–DNA adduct formation between CRL and FA-A cells at either the 3 or 7 μM dose. There was a slight but not statistically significant trend toward a higher adduct level in the CRL cells than in the FA-A cells. Three micromolar Cr(VI) resulted in approximately 0.02–0.03 Cr–DNA adducts per 10,000 base pairs. Seven micromolar Cr(VI) resulted in approximately 0.04–0.08 Cr–DNA adducts per 10,000 base pairs. Previous studies using a 10-fold higher concentration (75 μM) Cr(VI) resulted in approximately 2 Cr–DNA adducts/10,000 base pairs (27).

Discussion

The carcinogenic and transformagenic effects of Cr(VI) have been associated with

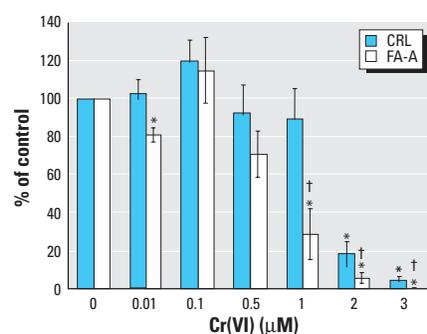


Figure 3. Effect of Cr(VI) on clonogenicity. CRL and FA-A cells exposed to 0, 0.01, 0.1, 0.5, 1, 2, or 3 μM Na_2CrO_4 for 24 hr were analyzed for cloning efficiency. The number of colonies for the indicated Na_2CrO_4 concentrations are expressed as a percentage of the 0 μM control for that cell line. Data represent an average (\pm SE) of three independent experiments, each done in triplicate. Asterisk (*) indicates a statistically significant difference ($p < 0.05$) relative to vehicle control. Cross (†) indicates a statistically significant difference ($p < 0.05$) between FA-A and CRL cells.

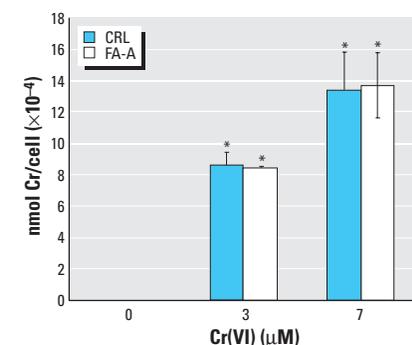


Figure 4. Measurement of ^{51}Cr (VI) uptake by CRL and FA-A cells. CRL and FA-A cells were treated with a final concentration of 0, 3, or 7 μM Na_2CrO_4 spiked with $\text{Na}_2\text{Cr}^{51}\text{O}_4$ for 3 hr at 37°C. Data represent an average (\pm SE) of three independent experiments, each done in triplicate. Asterisk (*) indicates a statistically significant difference ($p < 0.05$) relative to vehicle control.

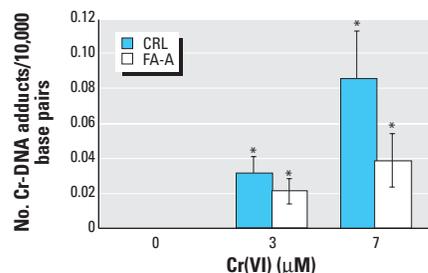


Figure 5. Measurement of ^{51}Cr (VI)-DNA adducts in CRL and FA-A cells. CRL and FA-A cells were treated with 0, 3, or 7 μM Na_2CrO_4 spiked with $\text{Na}_2\text{Cr}^{51}\text{O}_4$ for 2 hr at 37°C. Data represent an average (\pm SE) of three independent experiments, each done in duplicate. Asterisk (*) indicates a statistically significant difference ($p < 0.05$) relative to vehicle control.

the cytotoxicity of certain Cr(VI)-containing compounds at the site of administration *in vivo* (1) and in cell culture systems (12,29). In human occupational settings, exposure to levels of particulate Cr(VI) compounds usually associated with lung cancers also effect high levels of cell death, which manifest as nasal perforations and respiratory tract ulcerations (1). Several investigations have established the induction of apoptosis by Cr(VI) in treated normal human fibroblasts (29,42,43); thus, understanding the mechanisms of Cr-induced toxicity and cell death may help elucidate mechanisms of carcinogenicity.

The intracellular reduction of Cr(VI) generates several DNA-reactive species that can lead to myriad genetic lesions. Although a wealth of information exists concerning the genotoxicity of Cr(VI), the specific types of DNA damage that may be responsible for the toxic effects of Cr(VI) have not yet been determined. At least one type of genetic lesion formed as a result of Cr(VI) reduction (Cr-DDC) results in guanine-specific arrest of DNA replication (26,27). Although Cr-DDC may represent lethal lesions formed by Cr(VI), information is lacking on the effects of Cr(VI) on DNA cross-link repair-deficient cells. The focus of this investigation was to examine the cellular effects of Cr(VI) on FA-A cells, which are specifically deficient in the repair of DNA interstrand cross-links (35).

The ultimate fate of a cell exposed to a genotoxin such as Cr(VI) is heavily dependent upon the severity of the initial insult. At low concentrations, cells exposed to Cr(VI) primarily undergo growth arrest, presumably to allow time for repair. As the concentration of Cr(VI) is increased, the predominate cell fate becomes terminal growth arrest followed by apoptosis (29). The data from the present study are consistent with this model. The clonogenicity assay measures the long-term survival of a population after Cr(VI) exposure and accounts for both growth arrest and apoptosis. We found that low doses of Cr(VI) (0.1–3 μ M) produced a dramatic decrease in clonogenic survival (Figure 3). However, a marked increase in the number of apoptotic cells measured at a specific point in time [24 hr after the Cr(VI) exposure] was not detected at these doses (Figures 1, 2). These results suggest that terminal growth arrest, not apoptotic cell death, is the primary cell fate at this low Cr(VI) concentration, and that FA-A cells are more susceptible to undergo growth arrest at these low doses compared with control cells. At higher concentrations (6–7 μ M), FA-A cells demonstrated a statistically significant increase in apoptosis. These data show that FA-A cells are markedly hypersensitive to both the growth-inhibiting and apoptotic effects of

Cr(VI). These results are the first to show that FA cells are hypersensitive to an environmentally relevant DNA cross-linking agent.

The hypersensitivity of FA-A cells toward Cr(VI)-induced growth inhibition and apoptosis was not the direct result of higher Cr–DNA binding or Cr(VI) uptake. There were no differences found in Cr(VI) uptake or Cr–DNA adduct formation between FA-A and CRL cells. It is interesting however, that human FA-A fibroblasts were markedly hypersensitive to Cr(VI)-induced apoptosis compared with CRL cells. These data suggest that fewer Cr–DNA adducts (mono- and bifunctional) are required to induce equivalent growth arrest and apoptosis in FA-A cells relative to CRL cells. Additionally, the similar steady-state Cr–DNA adduct levels observed in this study suggest that either Cr–DNA adducts are resistant to removal in repair-competent cells or FA-A cells do not display a deficiency in Cr–DNA adduct removal.

Although the difference in Cr–DNA adduct levels between FA-A and CRL cells was not significant, it is interesting that there was a trend showing a decreased level of Cr–DNA adducts in FA-A cells exposed to relatively high doses of Cr(VI). This result may be caused by the marked increase in cytotoxicity in FA-A cells exposed to this dose of Cr(VI). The measurement of adduct levels and the magnitude of statistical error may be affected as the majority of cells progress through apoptosis. Alternatively, perhaps the lack of a functional DNA cross-link repair mechanism in FA-A cells causes an altered cellular response to Cr–DNA adduct formation. It may be possible that other molecular pathways designed to prevent or repair the formation of DNA adducts are upregulated in FA-A cells. Whatever the case, our results show that FA-A cells are more susceptible to Cr(VI)-induced growth arrest and apoptosis without exhibiting an increase in Cr-associated DNA.

Cr(VI) produces several types of DNA damage, and it is therefore possible that lesions other than Cr–DDC may contribute to the differential sensitivity of FA-A cells to chromium toxicity. FA-A cells are specifically deficient in repair of DDC, but one must consider the possible role of chromium-induced DPC (Cr–DPC) as well. We have shown that Cr–DPC are preferentially formed in and repaired from nuclear matrix DNA (44). Others have shown that the vast majority of Cr–DPC are actually individual amino acids or small peptides (such as glutathione) coordinated to DNA phosphate through ionic interactions (45), and that this lesion is premutagenic (46). We and others have found that this lesion correlates strongly with altered gene expression but not DNA replication (47). We have recently shown that DPC are not

polymerase-arresting lesions and that their formation actually precludes the formation of polymerase-arresting lesions (48). Combining information published by our laboratory and others, we can identify experimental manipulations that alter DPC and toxicity in an inverse fashion (14,21,43,49). Finally, although there is no information on FA-A cells and repair of DPC, we think it is unlikely that the mechanism of repair of this single-stranded, non-polymerase-arresting, complex mono-adduct would use a repair pathway designed to remove or bypass lethal DDC, which block progression of the replication fork.

In summary, human FA-A fibroblasts were markedly hypersensitive to Cr(VI)-induced apoptosis compared with CRL cells, although there were no differences found in Cr(VI) uptake or Cr–DNA adduct formation between FA-A and CRL cells. Because Cr(VI) produces Cr–DDC (24–27), and FA-A cells are known to be deficient in the repair of these lesions, these results suggest that Cr–DDC are terminal growth-arresting and/or proapoptotic lesions. We are currently exploring the differential formation and repair of polymerase-arresting lesions in FA-A and CRL cells.

REFERENCES AND NOTES

- IARC. Chromium, nickel and welding. IARC Monogr Eval Carcinog Risk Hum 49:1–648 (1990).
- Plunkett ER. Handbook of Industrial Toxicology. New York:Chemical Publishing, 1976.
- Amdur MO, Doull J, Klassen CD. Cassarett and Doull's Toxicology, 4th ed. New York:Maxwell-MacMillan-Pergamon, 1991.
- Ishikawa Y, Nakagawa K, Satoh Y, Kitagawa T, Sugano H, Hirano T, Tsuchiya E. Hot spots of chromium accumulation at bifurcations of chromate workers' bronchi. *Cancer Res* 54:2342–2346 (1994).
- Burke T, Fagliano J, Goldoft M, Hazen RE, Iglewicz R, McKee T. Chromite ore processing residue in Hudson County, New Jersey. *Environ Health Perspect* 92:131–137 (1991).
- Freeman NCG, Stern AH, Liou PJ. Exposure to chromium dust from homes in a chromium surveillance project. *Arch Environ Health* 52:213–226 (1997).
- Wise JP, Stearns D, Wetterhahn K, Patierno SR. Cell-mediated dissolution of carcinogenic lead chromate particles: role of individual dissolution products in clastogenesis. *Carcinogenesis* 15:2249–2254 (1994).
- Leonard A. Mechanisms in metal genotoxicity: significance of *in vitro* approaches. *Mutat Res* 198:321–326 (1988).
- Cohen M, Latta D, Coogan T, Costa M. Mechanisms of metal carcinogenesis: the reactions of metals with nucleic acids. In: *Biological Effects of Heavy Metals*, Vol 2 (Foulkes EC, ed). Boca Raton, FL: CRC Press, 1990:19–75.
- De Flora S, Bagnasco M, Serra D, Zancacchi P. Genotoxicity of chromium compounds. *Mutat Res* 238:99–172 (1990).
- Standeven A, Wetterhahn KE. Is there a role for oxygen species in the mechanism of chromium (VI) carcinogenesis? *Chem Res Toxicol* 4:616–625 (1991).
- Patierno SR, Banh D, Landolph JR. Transformation of C3H/10T1/2 mouse embryo cells to focus formation and anchorage independence by insoluble lead chromate but not soluble calcium chromate: relationship to mutagenesis and internalization of lead chromate particles. *Cancer Res* 48:5280–5288 (1988).
- Montaldi A, Zentilin L, Pagliulunga S, Levis AG. Solubilization by nitrotriacetic acid (NTA) of genetically active Cr(VI) and Pb(II) from insoluble metal compounds. *J Toxicol Environ Health* 21:387–394 (1987).

14. Wise JP, Orenstein J-M, Patierno SR. Inhibition of chromate clastogenesis by ascorbate: relationship to particle dissolution and uptake. *Carcinogenesis* 14:429–434 (1991).
15. Wise JP, Leonard JC, Patierno SR. Clastogenicity of lead chromate particles in hamster and human cells. *Mutat Res* 278:69–79 (1992).
16. Standeven AM, Wetterhahn KE. Chromium (VI) toxicity: uptake, reduction, and DNA damage. *J Am Cell Toxicol* 8:1275–1283 (1989).
17. Miller CA III, Cohen MD, Costa M. Complexing of actin and other nuclear proteins to DNA by *cis*-diamminedichloroplatinum (II) and chromium compounds. *Carcinogenesis* 12:269–276 (1991).
18. Miller CA III, Costa M. Characterization of DNA-protein complexes induced in intact cells by the carcinogen chromate. *Mol Carcinog* 1:125–133 (1988).
19. Cupo DY, Wetterhahn KE. Repair of chromate-induced DNA damage in chick embryo hepatocytes. *Carcinogenesis* 5:1705–1708 (1984).
20. Fornace AJ Jr, Seres DS, Lechner JF, Harris CC. DNA-protein cross-linking by chromium salts. *Chem Biol Interact* 36:345–354 (1981).
21. Sugiyama M, Tsuzuki K, Ogura R. Effect of ascorbic acid on DNA damage, cytotoxicity, glutathione reductase and formation of paramagnetic chromium in Chinese hamster V-79 cells treated with sodium chromate. *J Biol Chem* 266:3383–3383 (1991).
22. Sugiyama M, Patierno SR, Cantoni O, Costa M. Characterization of DNA lesions induced by CaCrO_4 in synchronous and asynchronous cultured mammalian cells. *Mol Pharmacol* 29:606–613 (1986).
23. Sugiyama M, Wang X-W, Costa M. Comparison of DNA lesions and cytotoxicity induced by calcium chromate in human, mouse and hamster cell lines. *Cancer Res* 46:4547–4551 (1986).
24. Xu J, Bublely GJ, Detrick B, Blankenship LJ, Patierno SR. Chromium (VI) treatment of normal human lung cells results in guanine-specific DNA polymerase arrest, DNA-DNA cross-links and S-phase blockade of cell cycle. *Carcinogenesis* 17:1511–1517 (1996).
25. Singh J, Bridgewater LC, Patierno SR. Differential sensitivity of chromium-mediated DNA interstrand crosslinks and DNA-protein crosslinks to disruption by alkali and EDTA. *Toxicol Sci* 45:72–76 (1998).
26. Bridgewater LC, Manning FC, Patierno SR. Base-specific arrest of in vitro DNA replication by carcinogenic chromium: relationship to DNA interstrand crosslinking. *Carcinogenesis* 15:2421–2427 (1994).
27. Bridgewater LC, Manning FC, Woo ES, Patierno SR. DNA polymerase arrest by adducted trivalent chromium. *Mol Carcinog* 9:122–133 (1994).
28. Evan G, Littlewood TA. A matter of life and cell death. *Science* 281:1317–1322 (1998).
29. Pritchard DE, Ceryak S, Ha L, Fornasaglio JL, Hartman SK, O'Brien TJ, Patierno SR. Mechanism of apoptosis and determination of cellular fate in chromium(VI)-exposed populations of telomerase-immortalized human fibroblasts. *Cell Growth Differ* 12:487–496 (2001).
30. Sasaki MA, Tonomura A. A high susceptibility of Fanconi anemia to chromosome breakage by DNA cross-linking agents. *Cancer Res* 33:1829–1836 (1973).
31. Strathdee CA, Buchwald M. Molecular and cellular biology of Fanconi anemia. *Am J Pediatr Hematol Oncol* 14:177–185 (1992).
32. Auerbach AD. Fanconi anemia diagnosis and the diepoxybutane (DEB) test. *Exp Hematol* 21:731–733 (1993).
33. D'Andrea AD, Grompe M. Molecular biology of Fanconi anemia: implications for diagnosis and therapy. *Blood* 90:1725–1736 (1997).
34. Buchwald M, Moustacchi E. Is Fanconi anemia caused by a defect in the processing of DNA damage? *Mutat Res* 408:75–90 (1998).
35. Zhen W, Evans MK, Haggerty CM, Bohr VA. Deficient gene specific repair of cisplatin-induced lesions in Xeroderma pigmentosum and Fanconi's anemia cell lines. *Carcinogenesis* 14:919–924 (1993).
36. Martin SJ, Reutlingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, Green DR. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiation stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 182:1545–1556 (1995).
37. Goldberg YP, Nicholson DW, Rasper DM, Kalchman MA, Koide HB, Graham RK, Bromm M, Kazemi-Esfarjani P, Thornberry NA, Vaillancourt JP, et al. Cleavage of Huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nat Genet* 13:442–449 (1996).
38. Nasir J, Goldberg YP, Hayden MR. Huntington disease: new insights into the relationship between CAG expansion and disease. *Hum Mol Genet* 5:1431–1435 (1996).
39. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA. Identification and inhibition of the ICE/CE3 protease necessary for mammalian apoptosis. *Nature* 376:37–43 (1995).
40. Rotonda J, Nicholson DW, Fazil KM, Gallant M, Garcau Y, Labelle M, Peterson EP, Rasper DM, Ruel R, Vaillancourt JP, et al. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. *Nat Struct Biol* 3:619–625 (1996).
41. Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Racie P, Flavell RA. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* 384:368–372 (1996).
42. Carlisle DL, Pritchard DE, Singh J, Patierno SR. Chromium(VI) induces p53 dependent apoptosis in diploid human lung and mouse dermal fibroblasts. *Mol Carcinog* 28:111–118 (2000).
43. Carlisle DL, Pritchard DE, Singh J, Owens BM, Blankenship LJ, Orenstein JM, Patierno SR. Apoptosis and p53 induction in human lung fibroblasts exposed to chromium (VI): effect of ascorbate and tocopherol. *Toxicol Sci* 55:60–68 (2000).
44. Xu J, Manning FCR, Patierno SR. Preferential formation and repair of chromium-induced DNA adducts and DNA-protein crosslinks in nuclear matrix DNA. *Carcinogenesis* 15:1443–1450 (1994).
45. Zhitkovich A, Voitkun V, Costa M. Glutathione and free amino acids form stable complexes with DNA following exposure of intact mammalian cells to chromate. *Carcinogenesis* 16:907–913 (1995).
46. Voitkun V, Zhitkovich A, Costa M. Cr(III)-mediated crosslinks of glutathione or amino acids to the DNA phosphate backbone are mutagenic in human cells. *Nucleic Acids Res* 26:2024–2030 (1998).
47. Manning FCR, Xu J, Patierno SR. Transcriptional inhibition by carcinogenic chromate: relationship to DNA damage. *Mol Carcinog* 6:270–279 (1992).
48. O'Brien T, Xu J, Patierno SR. Effects of glutathione on chromium-induced DNA crosslinking and DNA polymerase arrest. *Mol Cell Biochem* 222:173–182 (2001).
49. Blankenship LJ, Carlisle DL, Wise JP, Orenstein JM, Dye III LE, Patierno SR. Induction of apoptotic cell death by particulate lead chromate: differential effects of vitamins C and E on genotoxicity and survival. *Toxicol Appl Pharmacol* 146:270–280 (1997).